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14. ABSTRACT:

Instability at telomeres can result in "uncapping" of the ends of linear chromosomes, making them vulnerable to recombination, mutation and gross-chromosomal rearrangement (GCR). Continuously dividing human somatic cells and *S. cerevisiae* cells lacking functional telomerase experience progressive telomere degradation that culminates in replicative senescence. The disruption of telomere replication in *S. cerevisiae* leads to increases in deletions and mutations during replicative senescence at the *CAN1* locus that is located approximately 32kb from the telomere on the left arm of chromosome V. We show that the error-prone polymerase genes *RAD30*, *REV1 and REV7* control the stability of a telomere proximal locus, in the absence of telomere replication. We found that the absence of telomere replication led to a large increase in GCR during senescence. Furthermore, we show that *REV1* and *REV7* are necessary for increased GCR and mutation. Additionally, *RAD30* is involved in protecting telomere proximal sequences from GCR, potentially by promoting repair by homologous recombination. Our results demonstrate that error-prone polymerases are involved in maintaining the stability of telomere proximal sequences in the absence of telomerase. This suggests that DNA replication may be disrupted in these areas of the genome. This work changes the way we view the relationship between telomere dynamics and genome stability, and how these dynamics may contribute to cancer and genome evolution.

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Table of Contents

Introduction: Tumorigenesis is characterized by genome instability that results in genetic changes that promote a cancerous state[1]. Instability at telomeres can result in "uncapping" of the ends of linear chromosomes, making them vulnerable to recombination, mutation and gross-chromosomal rearrangement (GCR)[2,3,4]. Continuously dividing human somatic cells and *S. cerevisiae* cells lacking functional telomerase, a ribonucleoprotein complex required for telomere replication, experience progressive telomere degradation that culminates in replicative senescence[5,6]. The disruption of telomere replication in *S. cerevisiae* leads to increases in deletions and mutations during replicative senescence at the *CAN1* locus that is located approximately 32kb from the telomere on the left arm of chromosome V[2,3]. Here we show that the error-prone polymerase genes *RAD30, REV1 and REV7* control the stability of a telomere proximal locus, in the absence of telomere replication. We found that the absence of telomere replication led to a large increase in GCR during senescence. Furthermore, we show that *REV1* and *REV7* are necessary for increased GCR and mutation. Additionally, *RAD30* is involved in protecting telomere proximal sequences from GCR, potentially by promoting repair by homologous recombination[9]. Our results demonstrate that error-prone polymerases are involved in maintaining the stability of telomere proximal sequences in the absence of telomerase. This suggests that DNA replication may be disrupted in these areas of the genome. This work changes the way we view the relationship between telomere dynamics and genome stability, and how these dynamics may contribute to cancer and genome evolution.

Body:

Task 1: Creation of the mutant strains needed for the study.

a. I have created all relevant mutant strains needed for this study. The mutant strains created are *est2, est2, msh2, est2 mre11, est2 msh2 mre11, est2 msh2 rad51, est2 mre11 rad51, est2 msh2 mre11*-alleles and *est2 rad51 mre11*-alleles.

Task 2: Examine the role of Msh2 in restricting ALT in *S.cerevisiae.*

a. As shown in the previous annual report I have done both the serial liquid growth and viability assays to determine the number of generations until senescence.

b. Determining the advent of survivors in *est2* mutants and *est2 msh2* double mutants during the continual growth in liquid culture occurs during day 5 of liquid growth. This coincides with recovery of liquid growth cultures to near wild type growth density. The previous annual report stated the change in the ratio of typeI/typeII survivors in *est2 msh2* double mutants compared to *est2* mutants.

c. Currently I have not tested the triple *est2 msh2 rad51* and *est2 mre11 rad51*

mutants in order to focus on the role of error-prone polymerases in telomere stability in the absence of telomerase.

Task 3: Test the involvement of specific Mre11 domains in ALT.

a.-c. I have not yet started work on this phase of the research since I have focused on the role of error-prone polymerases in telomere stability in the absence of telomerase.

Recommended Changes/ Future Work:

In an effort to better understand the events that occur during senescence and the generation of survivors in *est2* mutants, three additional experiments are being done.

I. *Measuring the Frequency of Telomere Recombination.*

Currently there is no quantitative assay for examining spontaneous telomere recombination. Therefore, I have begun to construct an assay that will allow for the measurement of telomere recombination frequency. Determining the frequency of telomere recombination and its genetic control is critical in understanding ALT-dependent cancer progression. I plan on examining the role of Msh2 and Mre11 in telomere recombination during the progression toward senescence and subsequent survivors in *est2* mutants. Determining the frequency of telomere recombination will allow for a more sensitive measure of inhibition or enhancement of this process. This assay will allow for the identification of the best potential genetic targets for the inhibition of ALT.

The assay is shown below (Fig. 4, Appendices). The assay measures recombination between a pair of substrate sequences consisting of 3' and 5' truncated *HIS3* sequences at the end of chromosome V and the middle of chromosome III, respectively. We expect that telomere failure during replicative senescence will stimulate recombination between duplicate 500bp sequences in the 3' and 5' truncated *HIS3* substrates, generating an intact *HIS3* gene through ALT-mediated translocation. ALT will be measured by the appearance of His^+ colonies. I have completed all cloning steps needed for the creation of the telomere construct. Currently I am transforming the construct into the genome at which time I will test for proper insertion and for His+ colony formation.

II. *Measuring Genome Instability by Chromosome Loss, Interhomolog Recombination, Mutations and Gross Chromosomal Rearrangements.*

As *est2* mutants progress toward senescence it is thought that telomeres progressively shorten leading to a loss of "capping" and thus DNA end protection. As a result unfavorable genomic rearrangements can occur. This genomic instability also occurs in human somatic cells that have continued to divide beyond the normal control of cell growth. These cells are precursors in cancer development. Although most die, a few may potentially acquire genetic changes that allow them to progress toward a cancerous

state, which includes ALT. Therefore, it is of direct interest to study the genetic control of ALT and other genomic changes that accompany replicative senescence.

Among the genomic changes that accompany ALT during replicative senescence are deletions of chromosome ends called gross chromosomal rearrangements (GCR), and mutations of telomere linked genes. The GCR/mutation assay, shown below (Fig. 6, Appendices)[7], uses two markers, *CAN1* and *hxt13::URA3*, located 32kb and 21kb away from the telomere respectively. A detailed description of the GCR/mutation assay can be found in the attached manuscript in the methods section. I have recently found that these events are under the control of error prone polymerases suggesting that they are the result of DNA replication failure at telomere-linked loci during replicative senescence. This is also supported by examination of the mutation spectra in both wild type and *est2* mutant cells (Supplemental Table 2, Appendices) that shows no difference in the types of mutations that occur. We have also shown that *EXO1* is not required for the observed increases in GCR or *CAN1* mutation (Fig.7, Appendices). This suggests the observed increases in GCR and mutation is not due to Exo1-dependent exonuclease degradation. Finally, our examination of the mutation rate at the *CYH2* locus located 300kb from the telomere in both wild type and *est2* mutants (Supplemental Fig. 2, Appendices) showed no increase in the *CYH2* mutation rate in *est2* mutants during senescence relative to wild type. This suggests that the observed instability occurs at telomere proximal sequences and is not distributed throughout the genome.

Currently we are attempting to uncover the mechanism behind the increases in mutation and GCR by correlating them with increases in ssDNA at telomere linked loci. We suspect that senescence leads to a disruption of DNA replication near telomeres resulting in daughter strand gaps that ultimately stimulate mutation by GCR. We will detect ssDNA using a real-time PCR method called quantitative amplification of single-stranded DNA (QAOS)[10]. We are also attempting to perform the GCR/mutation assay using *est2 rad30 rev7* triple mutants to look for any epistatic relationships. Initial results show *est2 rad30 rev7* triple mutants have GCR rates similar to *est2 rad30* double mutants and *CAN1* mutation rate similar to *est2 rev7* mutants (see attached manuscript Figure 2 & 3).

Other studies in the lab suggest that disruptions in DNA replication such as those seen near telomeres in telomerase deficient cells significantly stimulate loss of heterozygosity in diploid cells. In turn, recombination between homologous chromosomes significantly improves the growth of replication defective cells. I am preparing to assay LOH and growth in *est2* mutant diploid cells. The assay is shown below (Fig. 5, Appendices), and makes use of two selectable markers on opposite ends of the centromere on chromosome V. Loss of both selectable markers will be counted as a chromosome loss event while loss of one marker will be counted as interhomolog recombination. Currently I have completed the construction of the LOH strains for wild type and *est2* mutants. In addition I am performing serial liquid growth and chromosome loss assays for diploid wild type and *est2* mutants. Initial results from the serial liquid growth assay of diploid homozygous *est2* mutants suggests a 30 generation delay in replicative senescence compared to haploid *est2* mutants. This suggests a potential growth advantage in diploids that lack functional telomerase that is due to the homolog. The chromosome loss results and liquid growth should be completed in 3-6 months.

Key Research Accomplishments:

- Generated an *est2::ura3::LEU2* that uses a new marker to follow *est2* mutants.
- Recapitulated results by Rizki & Lundblad (2001) showing *est2 msh2* cells grow better than *est2* during the time of senescence in liquid culture .
- Showed cell viability in *est2 msh2* double mutants is low but stable at around 25-35%, has a delayed recovery around 100-110 generations.
- Cell viability in *est2* mutants follow a similar pattern of cell growth, senescence and recovery observed in serial liquid growth.
- The relative ratio of Type II/I survivors in *est2* mutants is 23/50 = 0.46.
- The relative ratio of Type II/I survivors in *est2 msh2* double mutants is 33/1 = 33.
- Completed all cloning steps needed for the creation of the telomere construct.
- Created an *est2*/*est2* and wild type chromosome loss strain.
- Showed a 17-fold increase in the mutation rate of a telomere proximal gene in est2 mutants during the time of senescence. Furthermore, the mutation rate of a telomere proximal gene decreased to wild-type levels during survivor formation.
- Showed a 383-fold increase in the GCR rate in est2 mutants of telomere proximal sequences within 32kb of the telomere. This increased occurred during senescence and progressively decreased during the advent of survivor formation to wild type levels by ~125 generations.
- *est2 rev1* and *est2 rev7* double mutants show no increase in mutation or GCR during the time of senescence. This suggests a role of both Rev1 and Rev7 in generating GCR and mutation.
- *est2 rad30* mutants have a 37-fold increase in the GCR rate during the first testable time point relative to wild type. This enhanced increase is maintained during senescence to 2300-fold above wild type.
- Exo1 has no affect in the increases in GCR or mutation.
- Mutation increases during senescence of *est2* mutants does not occur in loci that are not telomere proximal.
- Mutation spectra are the same in wild type and *est2* mutants.

Reportable Outcomes: 1 manuscript (see attached).

Conclusions:

Understanding the events accompanying telomere destabilization and restabilization in *est2* mutants maybe critical to understanding the events leading to the development of certain cancers. This has been addressed by conducting GCR and mutation assays in *est2* mutants as they progressed toward and recovered from replicative senescence. Our results show a significant increase in both *CAN1* mutation and GCR in *est2* during the time of senescence and a subsequent decrease as survivors are generated. These results may shed light on the progression of cancer development in human cells. In human somatic cells, which lack telomerase, replicative senescence may stimulate mutation and genome rearrangement that accelerate the development of cancer.

"So What"

These results are helping us develop a better understanding of the progression of telomerase null somatic cells toward cancer. This may help to identify new molecular targets for drugs that kill these unique ALT cancer cells that would be immune to telomerase inhibitors.

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Control of GCR and Mutation by Error Prone Polymerases During Replicative Senescence in Telomerase Deficient Cells

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Tumorigenesis is characterized by genome instability that results in genetic changes that promote a cancerous state[1]. Instability at telomeres can result in "uncapping" of the ends of linear chromosomes, making them vulnerable to recombination, mutation and gross-chromosomal rearrangement (GCR)[2,3,4]. Continuously dividing human somatic cells and *S. cerevisiae* cells lacking functional telomerase, a ribonucleoprotein complex required for telomere replication, experience progressive telomere degradation that culminates in replicative senescence[5,6]. The disruption of telomere replication in *S. cerevisiae* leads to increases in deletions and mutations during replicative senescence at the *CAN1* locus that is located approximately 32kb from the telomere on the left arm of chromosome V[2,3]. Here we show that the error-prone polymerase genes *RAD30, REV1 and REV7* control the stability of a telomere proximal locus, in the absence of telomere replication. We found that the absence of telomere replication led to a large increase in GCR during senescence. Furthermore, we show that *REV1* and *REV7* are necessary for increased GCR and mutation. Additionally, *RAD30* is involved in protecting telomere proximal sequences from GCR, potentially by promoting repair by homologous recombination[9]. Our results demonstrate that error-prone polymerases are involved in maintaining the stability of telomere proximal sequences in the absence of telomerase. This suggests that DNA replication may be disrupted in these areas of the genome. This work changes the way we view the relationship between telomere dynamics and genome stability, and how these dynamics may contribute to cancer and genome evolution.

Initial work utilizing the GCR assay to examine telomerase deficient cells revealed no effect of the absence of telomere replication on the rate of GCR[7,8,10]. This work examined GCR in telomerase mutants before and after replicative senescence unlike recent reports that examined telomerase mutants serially over time[2,3]. Therefore, we examined GCR in mutants defective for *EST2,* which encodes the catalytic subunit of telomerase, serially over time. Serial liquid growth assays were performed to determine times of replicative senescence and recovery. The growth kinetics of the *est2* mutant and wild type cells (Fig. 1) were similar to those in previously published reports regarding initial cell growth, replicative senescence and recovery[2,3]. In simultaneous GCR and *CAN1* mutation rate assays (Fig. 2a, 3a) we observed no significant increase in the rates of GCR or *CAN1* mutation before senescence, consistent with previously published reports[3,8]. However, significant 17-fold ($p < 0.0001$) and 383-fold ($p < 0.0001$) increases in the rates of *CAN1* mutation and GCR were observed during senescence in *est2* mutant

cells (Fig. 2a, 3a). The senescence-dependent increase in the *CAN1* mutation rate is similar to that shown previously[3], however, the increase in GCR suggests an additional measure of genomic instability during replicative senescence in the absence of *EST2*. Continued growth of *est2* mutant cultures leads to a progressive decrease in the rates of *CAN1* mutation and GCR, that are ultimately, restored to wild type levels (Fig. 2a, 3a).

In order to determine whether the increase in mutation rate is restricted to telomere proximal loci the mutation rate at the *CYH2* locus, located 310 kb from the telomere, was determined. Wild type and *est2* mutant cells displayed no significant differences in the mutation rate at the *CYH2* locus before, during, or after replicative senescence (Supplementary information Fig. 2). This suggests that the observed increase in the *CAN1* mutation rate is restricted to telomere proximal sequences. Since the senescence-dependent increase in *CAN1* mutation rate occurs by an unknown mechanism we compared the mutation spectra of *CAN1* mutants obtained from wild type cells, and *est2* mutant cells experiencing replicative senescence. We observed no significant differences in the location or types of mutations in wild type and *est2* mutant cells (Supplementary information Fig. 3), suggesting that the mechanism involved in generating the mutations may be similar in both.

In an effort to better understand the mechanism underlying the senescence-dependent increase in *CAN1* mutation rate in *est2* mutant cells we examined the effects of mutations in genes that specify error prone polymerases, which play a role in the appearance of spontaneous mutations. The *RAD30* gene encodes pol η, a Y-family polymerase, that bypasses cyclobutane pyrimidine dimers and 8-oxoguanine lesions[11]. *REV7* encodes the structural subunit of the *REV3/7* B-family polymerase pol ζ, thought to extend from DNA lesions during mutagenic bypass[11]. Finally, *REV1* encodes the Rev1 polymerase that is a structural component required for pol ζ-dependent mutagenic bypass[11]. Determination of *CAN1* mutation rates from serial cultures of *rad30* single and *est2 rad30* double mutant cells revealed no significant difference from wild type and *est2* cultures at any time point (Fig. 2a, 2b). This shows that pol η does not contribute to the generation of *CAN1* mutations in wild type or *est2* mutant cells. Similarly, we observed no effect of the *rev1* and *rev7* mutations on the *CAN1* mutation rate in cells containing a functional *EST2* gene (Fig. 2a). However, the *rev1* and *rev7* mutations completely suppressed the stimulatory effect of the *est2* mutation in *est2 rev1* and *est2 rev7* double mutants, as the *CAN1* mutation rates were not significantly different from the *rev1* and *rev7* single mutants at any time point (Fig. 2a, b). No significant effects of the *rad30*, *rev1* or *rev7* mutations were observed on the kinetics of growth, senescence or recovery of *est2 rad30*, *est2 rev1*, or *est2 rev7* double mutants (Fig. 1) suggesting that the mutation of telomere-proximal sequences does not contribute to the initation of, or recovery from senescence. Taken together with previous data we suggest that pol ζ, but not pol η plays a role in the generation of mutations at telomere proximal loci in the absence of telomerase. This may occur through an increase in the spontaneous mutagenic bypass of DNA replication lesions that accumulate during replicative senescence.

We also found that *RAD30, REV1* and *REV7* play an important role in the control of GCR. While the *rad30* mutation alone has no significant effect on GCR, the *est2 rad30* double mutant displays a 37-fold ($p = 0.0006$) increase in GCR relative to wild type at the first time point (Fig. 3a, b). This increase continues through senescence reaching a level 2,673-fold ($p < 0.0001$) above wild type and then, ultimately returning to

wild type levels as the cultures recover (Fig. 3b). These results suggest that pol η plays a role in restricting GCR in telomerase deficient cells. Further, the elevated rate of GCR in the *est2 rad30* double mutant prior to senescence suggests that pol η attenuates GCR prior to substantial levels of telomere degradation. This argues that GCR may result from processes other than the erosion of chromosome ends in *est2* mutant cells. Since pol η is thought to be recruited to stalled replication forks[9], perhaps the loss of Est2 promotes the failure of bidirectional DNA replication at telomere-proximal loci, recruiting pol η. Pol η has been shown to extend D-loop intermediates *in* vitro[9], consistant with its involvement in homologous recombination[12]. We suggest that pol η may restrict GCR in *est2* mutant cells by promoting homologous recombination, which has previously been proposed to prevent GCR[13].

Unlike the loss of *RAD30*, loss of both *REV1* and *REV7* alone had significant effects on GCR as the rates in *rev1* and *rev7* single mutant cells were increased 5 to 10-fold over wild type. This suggests that pol ζ acts to suppress GCR in cells possessing functional telomerase. Strikingly, the *rev1* and *rev7* mutations are completely epistatic to *est2* with respect to GCR, as the GCR rates in the *est2 rev1* and *est2 rev7* double mutants are not significantly different from the *rev1* and *rev7* single mutants. Therefore, like mutation at the *CAN1* locus, senescence-dependent GCR requires pol ζ. While the mechanism by which pol ζ may promote GCR in senescent cells is unclear, we speculate that competition with pol η at stalled replication forks may be the basis. Pol ζ may outcompete pol η for stalled replication forks at telomere proximal loci in senescent cells, generating mutations and blocking pol η-dependent recombinational repair from limiting GCR. This is supported by the observation that the level of GCR in the *est2 rad30 rev7* triple mutant resembles the level in the *est2 rad30* double mutant (DM & AB, unpublished observations).

Our results demonstrate that telomere proximal loci are unstable in the absence of telomerase in yeast. Furthermore, this stability is linked to a dynamic relationship between error-prone polymerases that may respond to the failure of bidirectional DNA replication in the region[14,15]. Similar forces may underly some of the increased genome instability and cancer in somatic cells of elderly people, as human somatic cells lack telomerase[16,17]. The increased susceptibility of telomere proximal loci to senescence related instability might also suggest that these areas of the genome may have evolved to contain fewer essential genes. This and other ramifications of these observations are under active investigation.

Methods

Yeast Strains. All *S. cerevisiae* strains used are isogenic to W303. See supplemental table 1 for strain list.

Serial Liquid Growth Assay. Serial liquid growth was performed as describe previously[5]. Mean cell densities were reported and were calculated from at least eight independent colonies. Error bars represent two standard errors.

Serial GCR and *CAN1* Mutation Rate Assay. Fresh colonies of the appropriate genotype were taken in their entirety from the dissection plate, diluted in water and plated to YPD for viability, and medium containing canavinine for mutation rate determination. All assays were conducted at 30°C. Six serial calculations were performed for each

genotype (0-150 generations). *CAN1* mutation rate is calculated from the median *CAN1* mutation frequency of at least nine independent trials. GCR rate was determined by fluctuation analysis using at least 12 independent trials. Statistical significance was tested by determining the number of trials with each strain that were above and below the group median frequency, and then performing χ^2 analysis and Fisher's exact test. Representative *can*^{r} and *can*^{r} *ura3* colonies were subjected to Southern blot and genomic DNA sequence analysis.

Reference: 1. Michor, F., Iwasa, Y., Vogelstein, B., Lengauer, C. & Nowak, M.A. Can chromosomal instability initiate tumorigenesis? *Semin. Cancer Biol.* **15,** 43-49 (2005).

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Supplementary Information accompanies the paper on **www.nature.com/nature**. A figure summarizing the main result of this paper is also included as supplementary information Figure 1.

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Author Contributions D.M. performed all work relating to the paper. D.M. & A.B. contributed in writing the paper and discussion of results.

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Figure 1. Rev1, Rev7 and Rad30 do not significantly affect senescence and subsequent recovery. Results are the mean ±2SE from eight independent samples of each indicated genotype.

Figure 2. *REV1* and *REV7*, but not *RAD30* are required to observe increases in *CAN1* mutation rate during replicative senescence in *est2* mutant cells. **a**. *CAN1* mutation rate of wild type, *est2*, *rev1*, *rev7* and *rad30* mutant cells at the indicated time points. **b**. *CAN1* mutation rate of *est2 rev1*, *est2 rev7* and *est2 rad30* double mutant cells at the indicated time points. *CAN1* mutation rate was determined using the median *CAN1* mutation frequency from at least nine independent trials.

Figure 3. Polζ is required for GCR while pol η prevents GCR in *est2* mutant cells. **a**. Median GCR rate in wild type, *est2*, *rev1*, *rev7* and *rad30* mutant cells at the indicated generation time points. **b**. Median GCR rates in *est2 rev1*, *est2 rev7* and *est2 rad30* double mutants at the indicated time points. Median GCR rates were determined from at least 12 independent trials.

Figure 1a

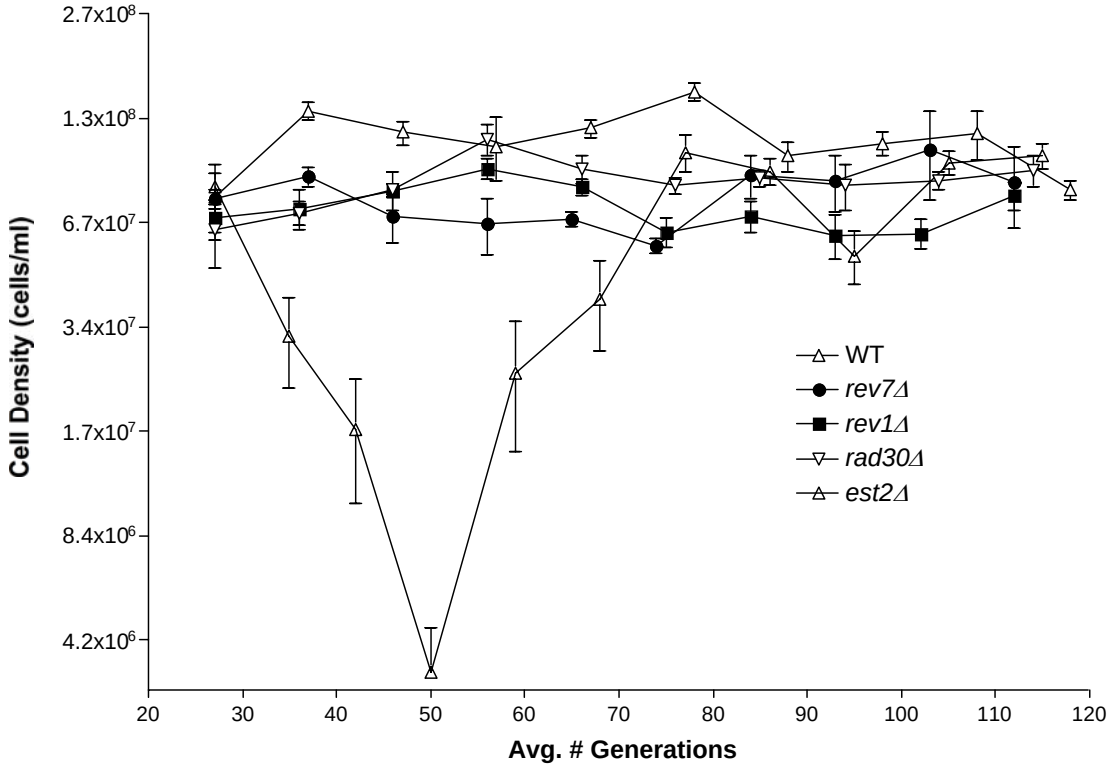

2.7x10^8
1.3x10^8
6.7x10^7
3.4x10^7
1.7x10^7
8.4x10^6
4.2x10^6
Cell Density (cells/ml)
20
30
40
50
60
70
80
90
100
110
120
Avg. # Generations
WT
rev7Δ
rev1Δ
rad30Δ
est2Δ

Figure 1b

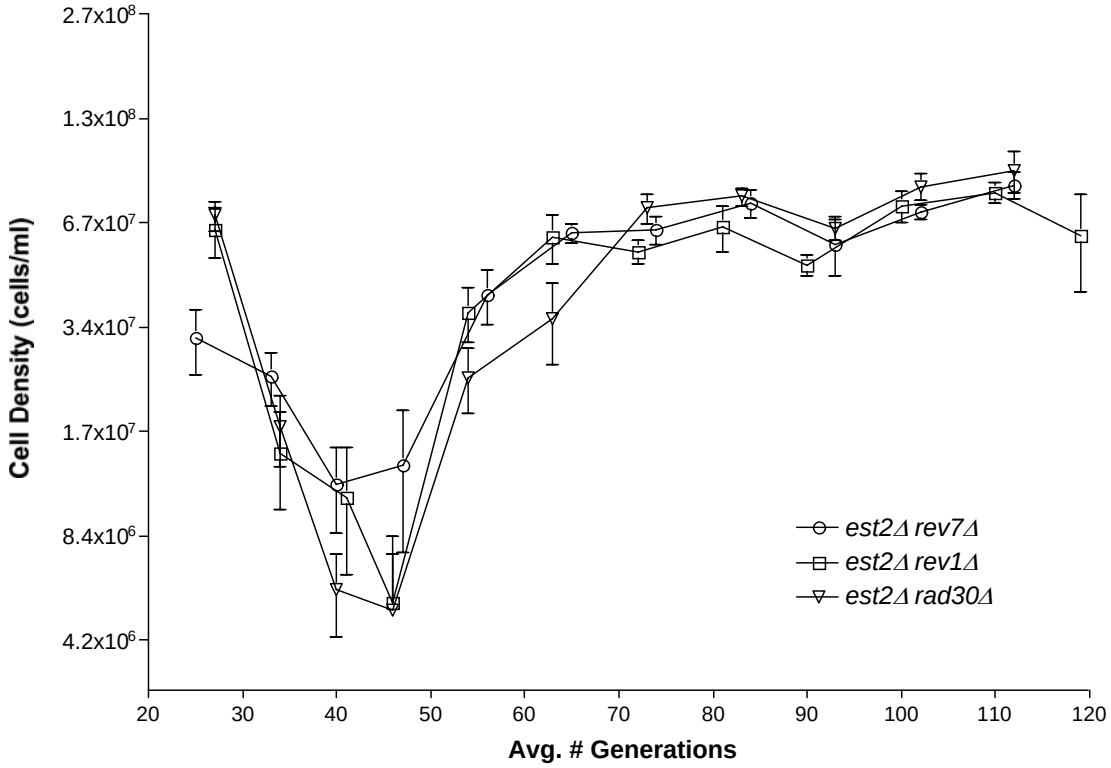

2.7x10^8
1.3x10^8
6.7x10^7
3.4x10^7
1.7x10^7
8.4x10^6
4.2x10^6
Cell Density (cells/ml)
20
30
40
50
60
70
80
90
100
110
120
Avg. # Generations
est2Δ rev7Δ
est2Δ rev1Δ
est2Δ rad30Δ

Figure 2a

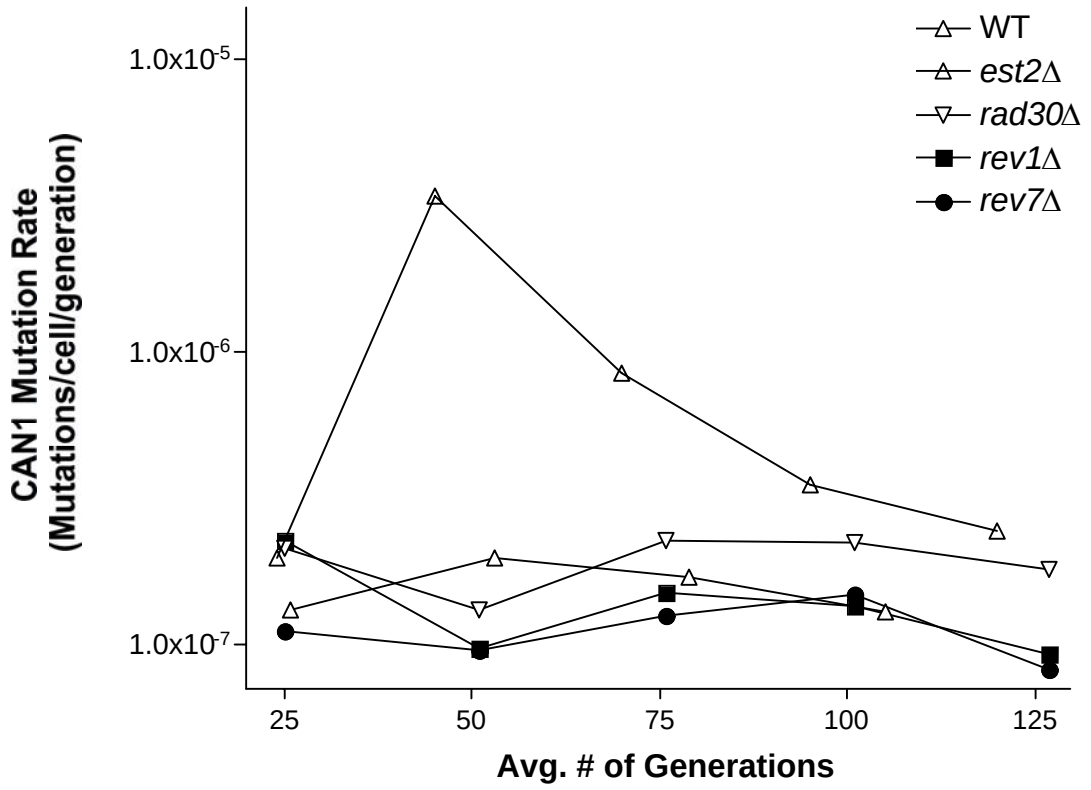

WT
est2Δ
rad30Δ
rev1Δ
rev7Δ
1.0x10-5
1.0x10-6
1.0x10-7
CAN1 Mutation Rate
(Mutations/cell/generation)
25
50
75
100
125
Avg. # of Generations

Figure 2b

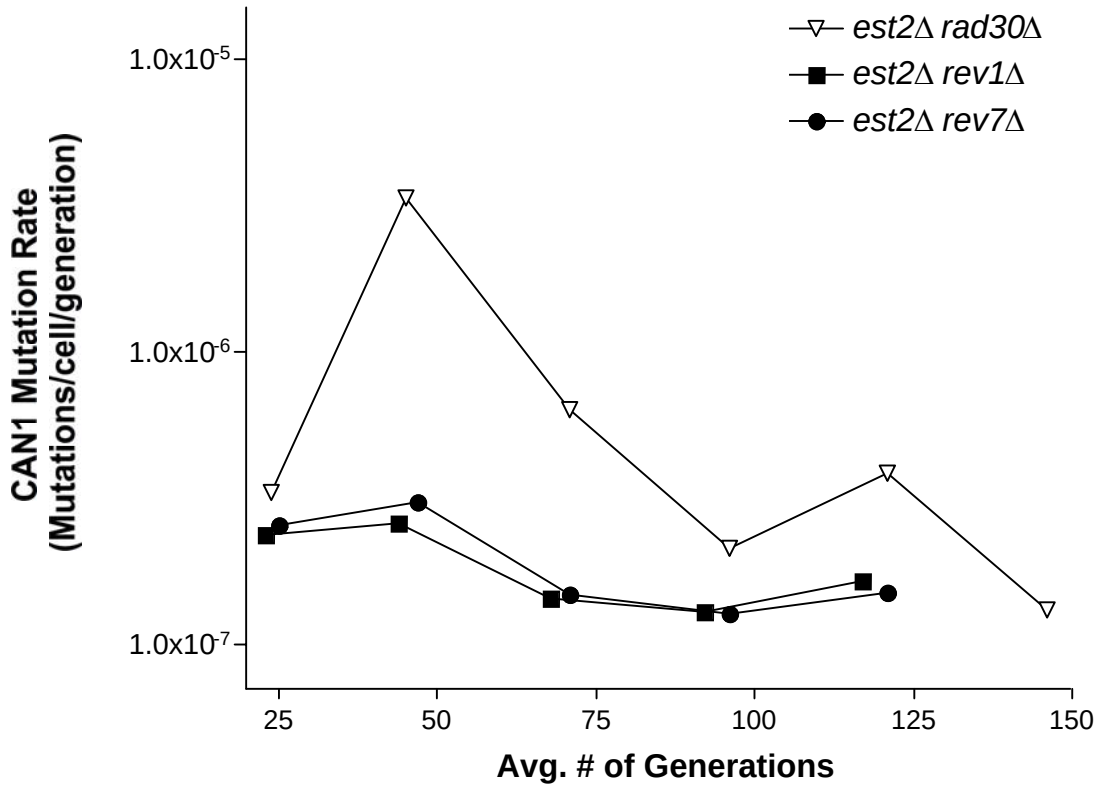

est2Δ rad30Δ
est2Δ rev1Δ
est2Δ rev7Δ
CAN1 Mutation Rate
(Mutations/cell/generation)
1.0x10-5
1.0x10-6
1.0x10-7
25
50
75
100
125
150
Avg. # of Generations

Figure 3a

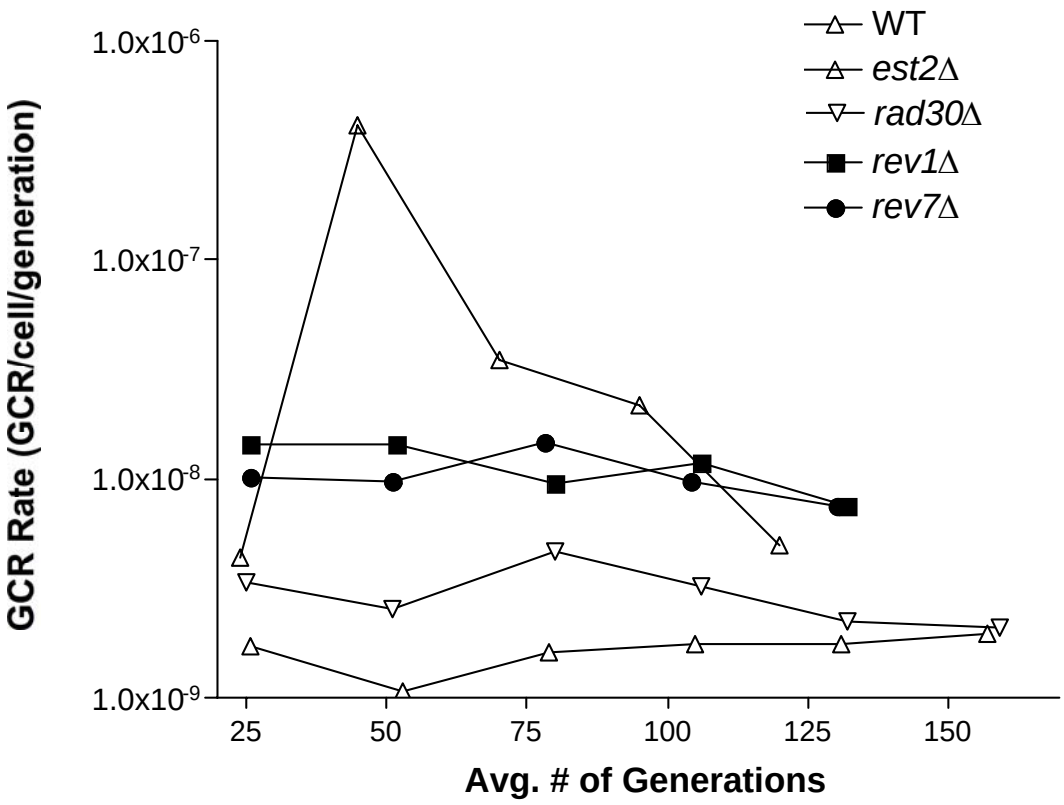

WT
est2Δ
rad30Δ
rev1Δ
rev7Δ
1.0x10-6
1.0x10-7
1.0x10-8
1.0x10-9
GCR Rate (GCR/cell/generation)
25
50
75
100
125
150
Avg. # of Generations

Figure 3b

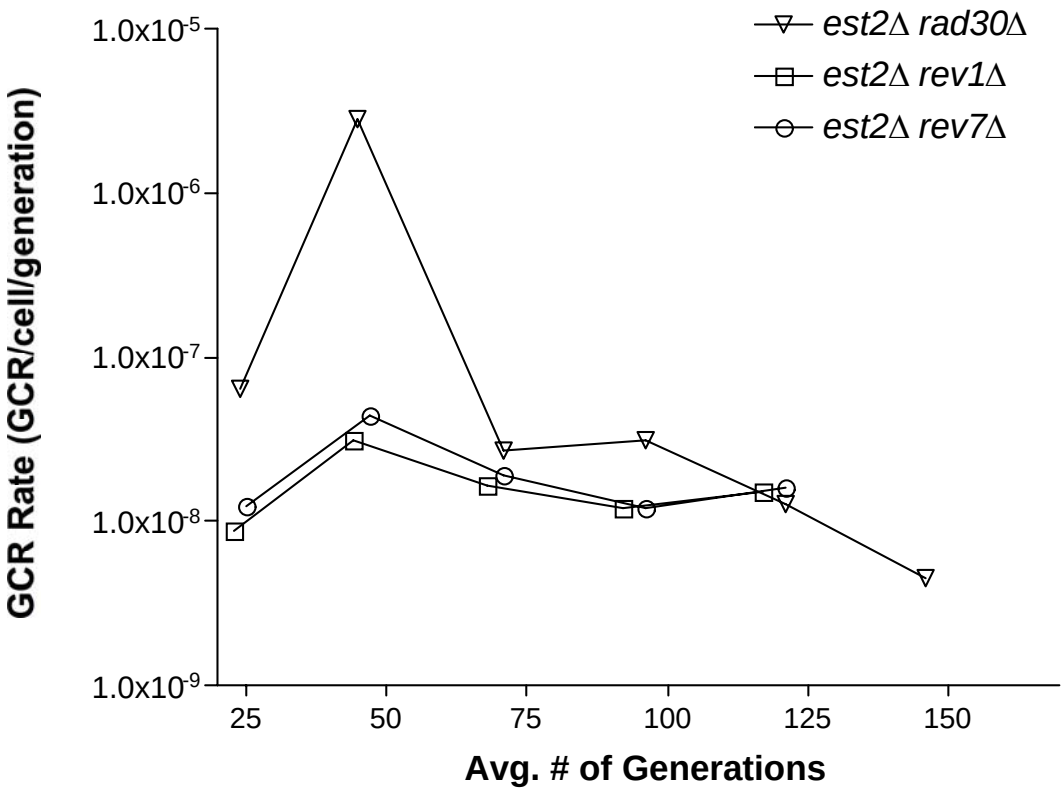

est2Δ rad30Δ
est2Δ rev1Δ
est2Δ rev7Δ
GCR Rate (GCR/cell/generation)
1.0x10-5
1.0x10-6
1.0x10-7
1.0x10-8
1.0x10-9
25
50
75
100
125
150
Avg. # of Generations

Figure 4 Telomere Recombination Assay

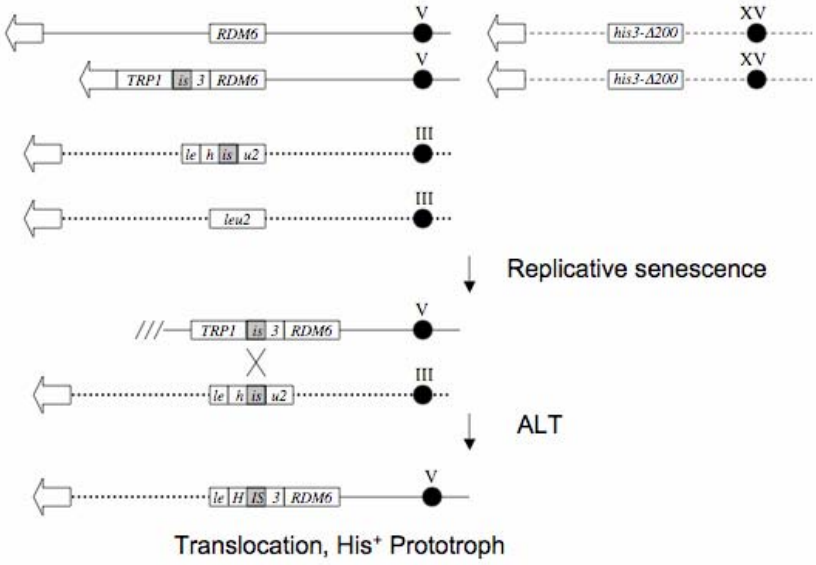


Figure 5 Chromosome Loss Assay

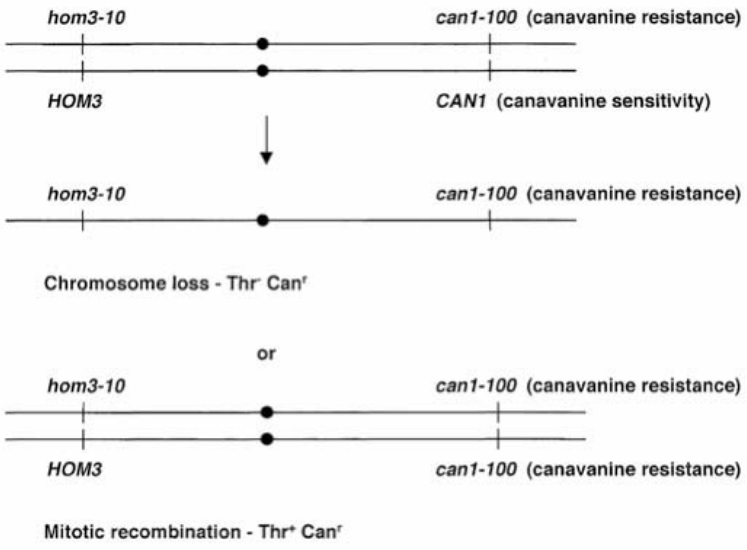


Figure 6 GCR and Mutation Assay

Figure 3. GCR and *CAN1* mutation assay. Loss of both *hxt13::URA3* and some part of *CAN1* leads to GCR. Any mutation in *CAN1* without loss of *hxt13::URA3* is a mutation.

Figure 7 *EXO1* is not Required for GCR or Mutation

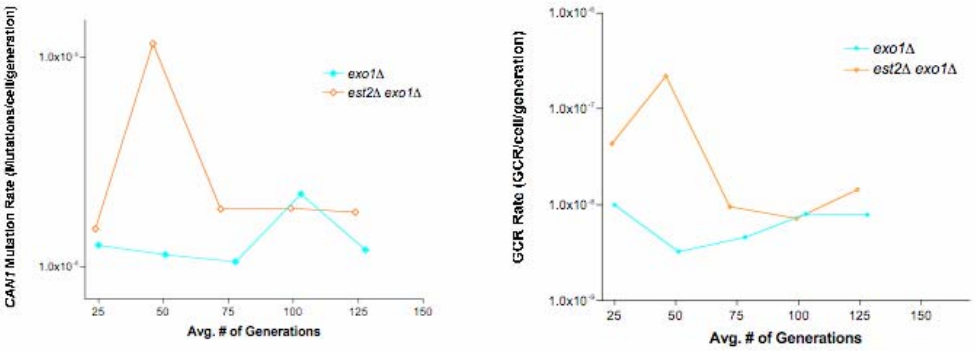
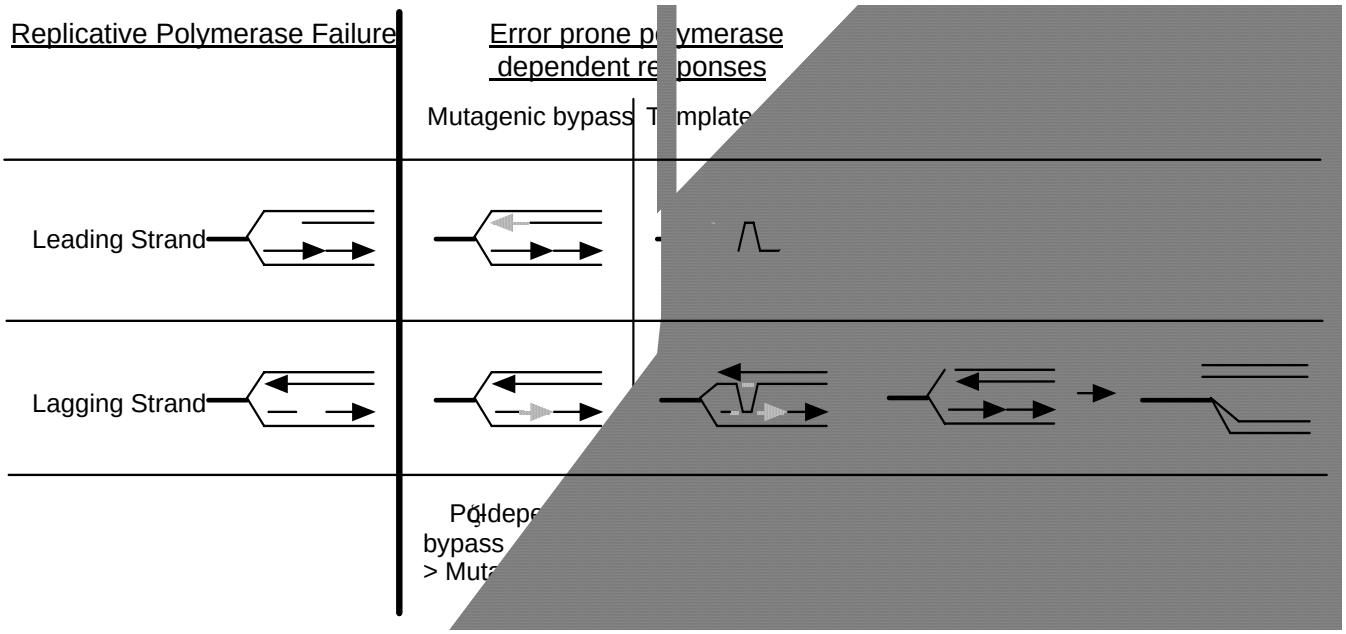
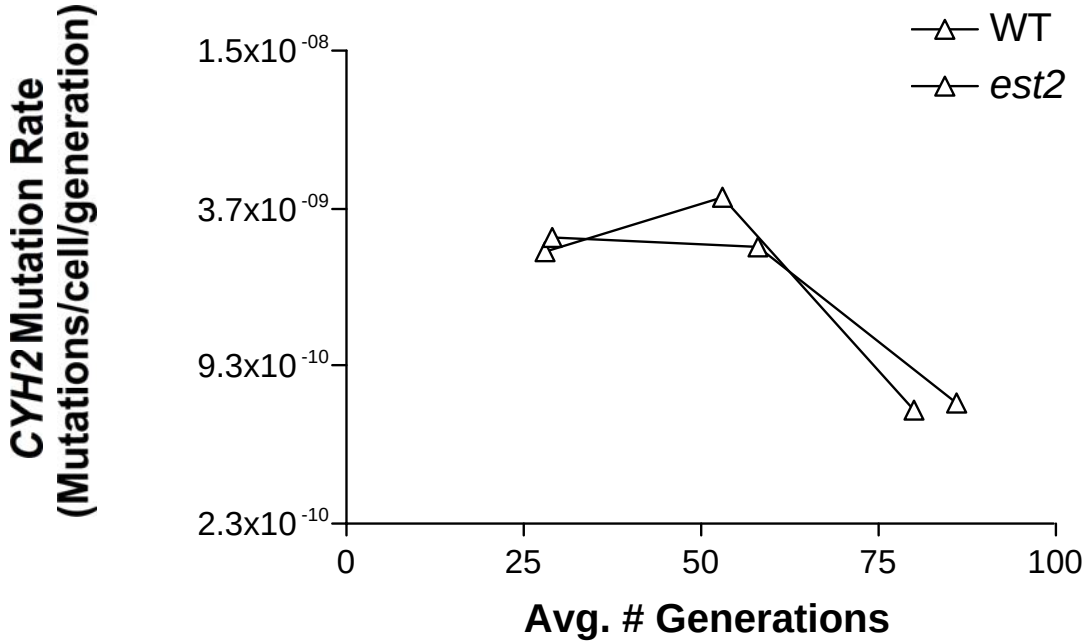


Figure 4. GCR and *CAN1* mutation rates for *exo1* and *est2 exo1* mutants. **A.** Mutation rate for *exo1* and *est2 exo1* mutants. **B.** GCR rate for *exo1* and *est2 exo1* mutants.



Supplementary Figure 1. Model summarizing the role of pol ζ and pol η in the events leading to GCR and mutation. The possible outcomes of error prone polymerase dependent and independent events in response to replicative polymerase failure on the leading strand and lagging strand.



Supplementary Figure 2. *CYH2* mutation rate does not increase in telomerase deficient cells during replicative senescence. Individual colonies of the appropriate genotype were selected at the indicated generation time and subject to *CYH2* mutation analysis.

Supplementary Table 1. *CAN1* Mutation Spectra in Wild Type and *est2* Mutant Cells

	Wild type	*est2*
Base Substitution	50%	50%
Frame Shift	43.75%	50%
Insertion/ Deletion	0%	0%
Complex	6.25%	0%

Supplementary Table 2. *S. cerevisiae* strains used in this study

Strain	*Genotype*
ABX1429	*MATα/a ade2-101/ ade2-1 can1-100/can1-100 leu2-Δ1/leu2-3,112 trp1-1/trp1-1 ura3-52/ ura3-1 his3-Δ200/ his3-11,15 adh4::URA3-TEL/ ADH4 CYH2/ CYH2 EST2/ est2::ura3::LEU2 RAD5/RAD5*
ABX1727	*MATα/**a** ade2-1/ade2-1 CAN1/ CAN1 HIS3/ his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3::TRP1/ura3::TRP1\ hxt13::URA3/ hxt13::URA3 EST2/ est2::ura3::LEU2 REV1/ rev1::HIS3 RAD5/RAD5*
ABX1729	*MATα/**a** ade2-1/ade2-1 CAN1/ CAN1 his3-11,15/ his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3::TRP1/ura3::TRP1 hxt13::URA3/ hxt13::URA3 EST2/ est2::ura3::LEU2 RAD30/rad30::HIS3 REV7/rev7::hisG RAD5/RAD5*